Differential Effects of Antiretroviral Nucleoside Analogs on Mitochondrial Function in HepG2 Cells

XIN-RU PAN-ZHOU,[1] LIXIN CUI,[1] XIAO-JIAN ZHOU,[1] JEAN-PIERRE SOMMADOSSI,[1]*
AND VICTOR M. DARLEY-USMAR[2]

Department of Clinical Pharmacology, Center for AIDS Research,[1] and Department of Pathology, Center for Free Radical Biology,[2] University of Alabama at Birmingham, Birmingham, Alabama 35294-0019

Numerous studies have reported effects of antiviral nucleoside analogs on mitochondrial function, but they have not correlated well with the observed toxic side effects. By comparing the effects of the five Food and Drug Administration-approved anti-human immunodeficiency virus nucleoside analogs, zidovudine (3′-azido-3′-deoxythymidine) (AZT), 2′,3′-dideoxycytidine (ddC), 2′,3′-dideoxyinosine (ddI), 2′,3′-didehydro-2′,3′-deoxythymidine (d4T), and β-L-2′,3′-dideoxy-3′-thiacytidine (3TC), as well as the metabolite of AZT, 3′-amino-3′-deoxythymidine (AMT), on mitochondrial function in a human hepatoma cell line, this issue has been reexamined. Evidence for a number of mitochondrial defects with AZT, ddC, and ddI was found, but only AZT induced a marked rise in lactic acid levels. Only in mitochondria isolated from AZT (50 μM)-treated cells was significant inhibition of cytochrome c oxidase and citrate synthase found. Our investigations also demonstrated that AZT, d4T, and 3TC did not affect the synthesis of the 11 polypeptides encoded by mitochondrial DNA, while ddC caused 70% reduction of total polypeptide content and ddI reduced by 43% the total content of 8 polypeptides (including NADH dehydrogenase subunits 1, 2, 4, and 5, cytochrome c oxidase subunits I to III, and cytochrome b). We hypothesize that in hepatocytes the reserve capacity for mitochondrial respiration is such that inhibition of respiratory enzymes is unlikely to become critical. In contrast, the combined inhibition of the citric acid cycle and electron transport greatly enhances the dependence of the cell on glycolysis and may explain why apparent mitochondrial dysfunction is more prevalent with AZT treatment.

The use of nucleoside analogs for the treatment of human immunodeficiency virus (HIV) infection now includes a spectrum of compounds, such as zidovudine (3′-azido-3′-deoxythymidine) (AZT), 2′,3′-dideoxycytidine (ddC), 2′,3′-dideoxyinosine (ddI), 2′,3′-didehydro-2′,3′-deoxythymidine (d4T), and β-L-2′,3′-dideoxy-3′-thiacytidine (3TC). Some of these compounds have been associated with side effects that have been ascribed to the induction of mitochondrial dysfunction. While it is well recognized that the dose-limiting toxicity for AZT therapy is hematological, long-term treatment has also been shown to induce mitochondrial defects. This is manifested most commonly as a myopathy, with the appearance of ragged red fibers, depletion of muscle mitochondrial DNA (mtDNA), and a partial cytochrome c oxidase (COX) deficiency (10, 16, 29, 33). With other nucleosides, mitochondrial defects that are thought to contribute to peripheral neuropathy and pancreatitis are apparent. These responses are the major toxicities associated with treatment of HIV infection by ddC, d4T, and ddI and emphasize the importance of understanding the basis of mitochondrial defects and the differential sensitivities observed for these tissues (6, 20, 45).

Importantly, the emergence of hepatic failure with type B lactic acidosis is a variable response to treatment with this class of antiviral compounds, notably FIAU [1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil] (21, 43). An analogous toxic side effect has been reported in response to AZT treatment, but this is an unusual syndrome with an estimated incidence of 1.3 per 1,000 person-years of follow-up in patients treated with a cohort of antiretroviral nucleoside analogs (22). This severe

toxicity, while not as common as that found with FIAU, is important to understand. Liver biopsies of these patients showed massive macrovesicular steatosis and enlarged irregular mitochondria under electron microscopy (41). The association of macrovesicular steatosis and liver failure after nucleoside analog therapy is regarded as a unique clinical circumstance (41). This particular hepatotoxicity has also been reported in obese women who had received AZT for at least 6 months (23). Interestingly, these data also suggest that a mitochondrial defect underlies this toxicity with an undue reliance on glycolysis for the synthesis of ATP.

The underlying mechanism(s) responsible for nucleoside analog-induced mitochondrial abnormalities is not completely understood. Prior to exerting antiviral activities, nucleoside analogs need to be phosphorylated to their respective 5′-triphosphates. These metabolites are thought to competitively inhibit the viral reverse transcriptase or to be incorporated into the viral genome, causing termination of viral DNA chain elongation (24, 30). Although the 5′-triphosphates of these nucleoside analogs have less affinity for most of the human cellular nuclear DNA polymerases (α, β, δ, and ϵ), ddC, d4T, and 2′,3′-dideoxyadenosine triphosphates are potent inhibitors of DNA polymerase γ. This is significant, since this is the only DNA polymerase located inside the mitochondrial matrix and it is responsible for mtDNA synthesis (28). AZT triphosphate is a 20- to 2,000-fold-less-potent inhibitor of DNA polymerase γ than ddC triphosphate. However, the accumulation of AZT monophosphate may inhibit the exonuclease and contribute to the toxicity of AZT by interfering with the repair of AZT-terminated DNA (5). Among the five nucleoside analogs, 3TC exhibits minimal cytotoxicity, which has been attributed to the lack of inhibition of DNA polymerase γ and therefore lack of disruption of mtDNA synthesis (25). Nevertheless, the interaction of nucleoside analog triphosphates with DNA polymer-

* Corresponding author. Mailing address: University of Alabama at Birmingham/Box 600, Volker Hall GO19, University Station, Birmingham, AL 35294. Phone: (205) 934-8266. Fax: (205) 975-4871. E-mail: Jean-Pierre.Sommadossi@ccc.uab.edu.

ase γ may lead to reduced mtDNA replication, resulting in further mitochondrial dysfunction.

Recent insights into mitochondrial function have revealed the fact that profound inhibition of respiratory complexes may have little or no effect on the capacity of the organelle to synthesize sufficient ATP for the cell's requirements (18). There is then a substantial reserve metabolic capacity in the mitochondria, and each respiratory chain can be inhibited to a substantial degree before oxidative phosphorylation is affected. The point at which inhibition results in decreased synthesis of ATP may be tissue and cell specific and is then the "threshold" for that respiratory complex in the control of mitochondrial function. This is particularly intriguing with respect to the effects of nucleoside analogs on mitochondrial function. For example, on the basis of histochemical studies of patients (10), AZT is thought to inhibit COX. This enzyme in the respiratory chain is not rate limiting, and loss of activity in excess of 60 to 90% would probably be required before a significant effect on mitochondrial ATP synthesis occurred (19). It thus appears likely that multiple sites of inhibition of mitochondrial function are required before significant changes in respiratory metabolism can occur. To address this issue, mitochondrial function was assessed in hepatocytes exposed to a range of nucleoside analogs commonly used in HIV therapy. Mitochondrial function was assessed in the presence of nucleosides at multiple levels, including morphology, DNA synthesis, and synthesis of mitochondrial polypeptides. In addition, oxidative phosphorylation and the activity of the tricarboxylic acid cycle enzyme citrate synthase were evaluated with selected compounds.

MATERIALS AND METHODS

Materials. The HepG2 cell line was purchased from the American Type Culture Collection. Minimum essential medium with nonessential amino acids, sodium pyruvate, fetal bovine serum, and 10× trypsin-EDTA were purchased from Gibco Life Technologies, Inc. AZT, AMT (3'-amino-3'-deoxythymidine), ddC, ddI, d4T, decylubiquinone, antimycin, rotenone, dodecyl-maltoside, NADH, and 2,6-dichlorophenolindophenol were obtained from the Sigma Chemical Co. 3TC was a gift from R. F. Schinazi (Department of Pediatrics, Emory University, Decatur, Ga.). Cytochrome c, [^{35}S]methionine (1 mCi/mmol; 37 MBq/mmol), [α-^{32}P]dATP (3,000 Ci/mmol), and [α-^{32}P]dCTP (3,000 Ci/mmol) were purchased from ICN Biomedicals, Inc. QuickHyb hybridization solution was purchased from Stratagene. A lactic acid assay kit was purchased from Boehringer Mannheim Corp.

Determination of cytotoxicity and L-lactic acid and morphological evaluation. HepG2 cells (2.5 × 10^4/ml) were plated into 12-well cell culture clusters in minimum essential medium with nonessential amino acids supplemented with 10% fetal bovine serum, 1% sodium pyruvate, and 1% penicillin-streptomycin and exposed to various concentrations of AZT, ddC, ddI, d4T, 3TC, and AMT for 4 days. The culture medium and drugs were renewed every 2 days. On day 6, cell numbers were determined with a hemocytometer by counting viable cells by the trypan blue exclusion method on trypsinized cells. The level of lactic acid in the medium after 4 days of treatment was determined with a Boehringer lactic acid kit and was expressed as milligrams of lactic acid production per 10^6 cells. Electron microscopic evaluation of mitochondrial morphological changes was performed as described previously (14). Briefly, the cells were fixed in 1% glutaraldehyde for 1 h, washed with sodium phosphate buffer, and postfixed in 1% osmium tetroxide for 1 h. The cells were gradually dehydrated with increasing concentrations of ethanol from 50% through 100% in propylene oxide and slowly infiltrated and embedded in epon. The cells were then sectioned with a Reichert-Jung ultramicrotome and stained with uranyl acetate and lead citrate. Finally, the cells were examined with a Hitachi 7000 electron microscope.

Effect of nucleoside analogs on mtDNA content. HepG2 cells (2.5 × 10^4/ml) were exposed to different concentrations of AZT, ddC, ddI, d4T, 3TC, and AMT for 14 days. Previous studies of the effects of nucleoside analogs on mtDNA content conducted in our laboratory showed effects after exposure for 14 days, and this time period was selected for the studies of function. The cells (5 × 10^4) were heated at 100°C for 10 min in 0.5 ml of 0.4 M NaOH and 10 mM EDTA buffer. DNA was slot blotted onto a Zeta-Probe membrane. The [α-^{32}P]dATP-labeled specific human oligonucleotide mitochondrial probe encompassing nucleotide positions 4212 to 4242 was used at 2.5 × 10^6 dpm/ml. Prehybridization, hybridization, and washes were performed with QuickHyb hybridization solution according to the manufacturer's instructions. The amount of total cellular DNA loaded on the membrane was standardized with a 625-bp fragment of a human β-actin cDNA plasmid probe as previously described (14). The amount of

mtDNA was determined as a ratio of the oligonucleotide probe radioactive signal to the β-actin probe radioactive signal.

Isolation of mitochondria and enzyme assays. HepG2 cells (20 × 10^6) were grown in a 175-cm^2 tissue culture flask. Various concentrations of AZT, AMT, ddC, d4T, or buffer with no nucleoside analogs (control) were added to the medium, which was changed every 2 days. After 6 days, the cell monolayers were treated with 10% trypsin–phosphate-buffered saline (PBS) and then neutralized with the medium. The cells were washed three times with cold PBS and then suspended in 4 ml of 10 mM triethanolamine acetate buffer, pH 7.4, containing 0.25 M sucrose, 1 mM EGTA, and 0.5% bovine serum albumin. Isolation of mitochondria was performed according to the method of Rickwood et al. (34). Briefly, the cells were homogenized with a glass homogenizer and centrifuged twice at 1,300 × g for 10 min at 4°C. The supernatant was centrifuged at 12,000 × g for 10 min. The pellets (mitochondria) were washed three times and resuspended in 10 mM Tris-HCl, pH 7.4, containing 10 mM KCl, 0.25 M sucrose, and 5 mM MgCl$_2$ and stored at −70°C. Protein was measured as described by Bradford (4). The contamination of microsomes was examined by determining glucose-6-phosphatase activity in mitochondrial fractions compared with that in cell homogenates and was found to be less than 5% (7).

The activities of complexes I, II, III, and IV were measured as previously described (3). Briefly, 20 μg of freeze-thawed mitochondrial protein was added to the complex I reaction mixture (25 mM potassium phosphate [pH 7.2], 5 mM MgCl$_2$, 2.5 mg of bovine serum albumin/ml, 5 mM KCN, 0.13 mM NADH, 65 μM ubiquinone, 2 μg of antimycin A/ml). Complex I activity was measured through the decrease in absorption at 340 nm of NADH oxidation with decylubiquinone as an electron acceptor in the presence or absence of rotenone (ε = 6.81 mM^{-1} cm^{-1}). Complex II activity was assayed by measuring a decrease in absorbance at 600 nm for the rate of reduction of 2,6-dichlorophenolindophenol (ε = 19.1 mM^{-1} cm^{-1}). Complex III activity was measured by the reduction of cytochrome c at 550 nm and was expressed as the first-order rate constant per milligram of protein. Complex IV activity was measured by following the oxidation of reduced cytochrome c at 550 nm and was expressed as the first-order rate constant per milligram of protein.

Citrate synthase activity was recorded spectrophotometrically at 412 nm (35). A background rate was obtained by adding freeze-thawed mitochondria (30 μg of protein) to 0.1 mM 5,5'-dithiobis-2-nitrobenzoate and 0.3 mM acetyl-coenzyme A. This initial rate was subtracted from the rate obtained upon the addition of the substrate (0.1 mM oxaloacetate). All enzyme assays were performed at 30°C in a final volume of 1 ml with a Perkin-Elmer Lambda 6 spectrophotometer.

Autoradiography of radiolabeled mitochondrially encoded proteins. HepG2 cells were treated with 50 μM AZT, ddI, d4T, or 3TC and 10 μM ddC for 6 days. At day 6, the medium was replaced by a medium without methionine but with 200 μg of emetine/ml and 60 μCi of [^{35}S]methionine/ml. After incubation at 37°C for 3 h, the cells were washed three times with PBS and dissolved in 5% sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulfonyl fluoride, and 10 mM Tris-HCl, pH 6.5. Protein was measured with a Bio-Rad DC protein assay kit. Equal amounts of mitochondrial proteins (40 μg) were run on a urea–SDS–15% polyacrylamide gel with 2,5-diphenyloxazole-mediated fluorography (17). The dried gel was exposed to X-ray film at −70°C for 4 days.

RESULTS

Cytotoxicity and evaluation of mitochondria by electron microscopy in HepG2 cells treated with nucleoside analogs. After treatment with various concentrations of nucleoside analogs for 6 days, AMT had the most profound effect on cell proliferation, with a 50% inhibitory concentration of about 7 μM. The corresponding values for AZT and ddC were 14 and 20 μM, respectively, whereas d4T, ddI, and 3TC were not toxic to HepG2 cells at concentrations up to 50 μM. Following 14 days of exposure to AZT, AMT, ddC, d4T, and 3TC, the ultrastructures of HepG2 cells were examined by electron microscopy. No discernible changes in cell structure were observed with d4T and 3TC. In contrast to the studies with anti-hepatitis B compounds such as FIAU, none of the drug treatments resulted in an increase in lipid droplet formation compared with the untreated control. With respect to mitochondria, only AZT slightly enlarged the organelle size, whereas mitochondrial cristae appeared to have been disrupted in ddC- and ddI-treated cells (Fig. 1).

Effect of nucleoside analogs on the mtDNA content and extracellular lactic acid levels. Exposure of HepG2 cells for 14 days to AZT, AMT, d4T, and 3TC at concentrations up to 50 μM resulted in no deleterious effect on mtDNA levels (Fig. 2). In contrast, ddC at 1 μM and ddI at the high concentration of 200 μM decreased mtDNA levels by 85%. These data are
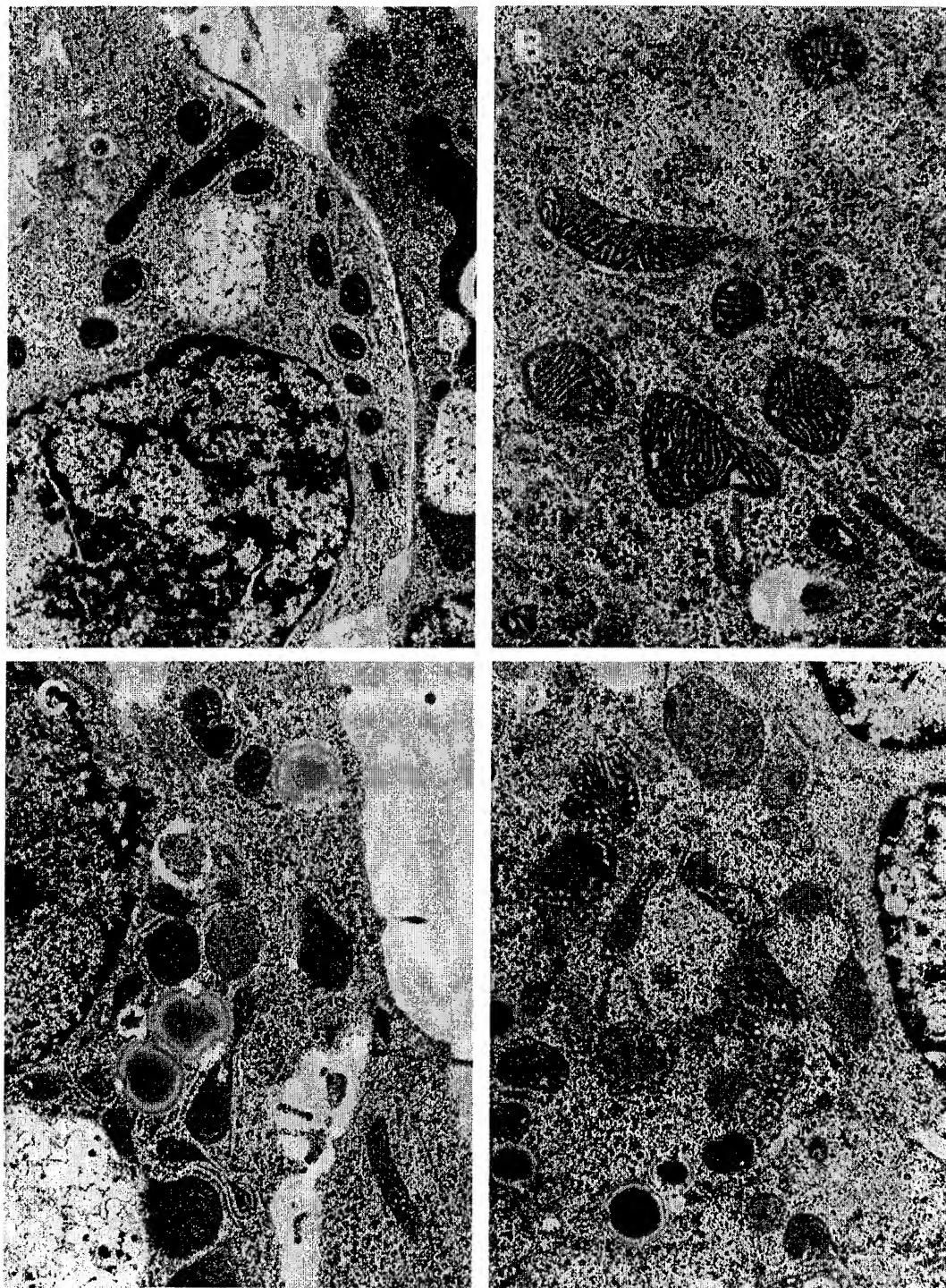
FIG. 1. Electron micrograph of HepG2 cells treated with nucleosides. After 14 days of incubation of HepG2 cells with the control (A), 10 μM AZT (B), 50 μM ddI (C), and 10 μM ddC (D), electron microscopy was undertaken (magnification, ×30,000). Disrupted mitochondrial cristae were observed in ddC- and ddI-treated cells.
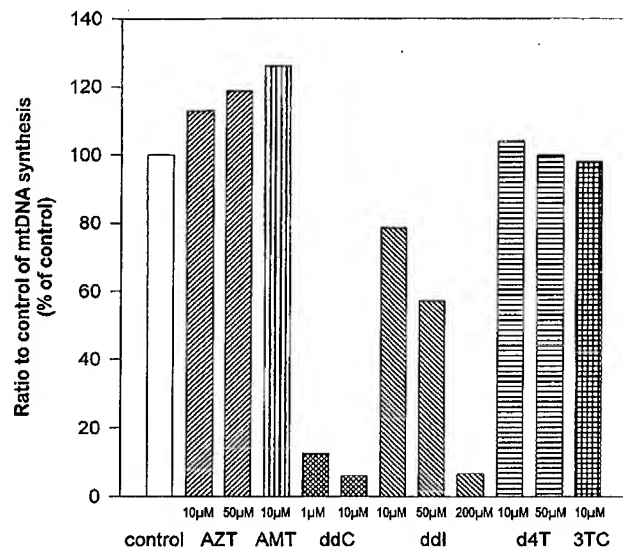
FIG. 2. Effects of nucleoside analogs on mtDNA content of HepG2 cells. HepG2 cells (3×10^4/ml) were treated with various concentrations of nucleoside analogs for 14 days or were not given drug treatment (control). The mtDNA of HepG2 cells on the nitrocellulose paper was detected by using ^{32}P-labeled human mtDNA fragment as described in Materials and Methods. The data represent the means of two experiments.
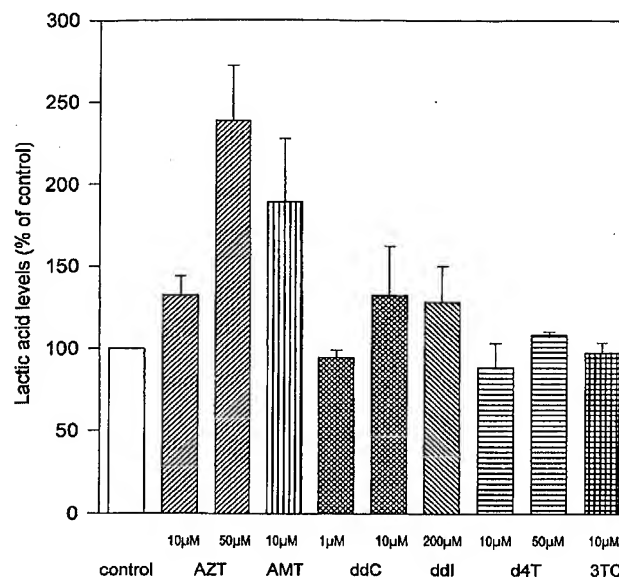

FIG. 3. Levels of lactic acid in the extracellular medium of HepG2 cells after treatment with various concentrations of nucleoside analogs for 4 days. Lactic acid levels are expressed as milligrams of lactic acid per 10^6 cells. The lactic acid levels at each drug concentration were compared with those in cells incubated in drug-free medium, and the percentage of the control levels was determined for each concentration. The standard deviations (error bars) of the data were determined from the means of three experiments.

consistent with previous studies using ddC and ddI with other cell types (11, 13) and indicate that ddC- and ddI-dependent inhibition of mtDNA replication is probably not cell specific.

Lactic acid production can be considered a marker of impaired mitochondrial function. As shown in Fig. 3, the level of lactic acid in the medium was markedly increased, approximately 240 and 190%, respectively, by AZT (50 μM) and AMT (10 μM) treatment. In contrast the medium of cells treated with d4T, 3TC, ddC, or ddI had essentially the same lactic acid level as that of control cells. These results demonstrated a lack of direct correlation between the increase in medium lactic acid and inhibition of mtDNA replication by these nucleoside analogs. This suggests that, at this level of exposure, the inhibitory effects of ddC on mtDNA were not yet evident in terms of oxidative phosphorylation. This could occur if the turnover of mitochondrially coded proteins is slow relative to the period of the experiment (14 days) or DNA levels are not limiting for the synthesis of new protein.

Effects of nucleoside analogs on mitochondrial protein synthesis. To determine whether mitochondrial protein synthesis was affected by these nucleoside analogs, 13 mitochondrially encoded polypeptides were labeled by [^{35}S]methionine in the presence of emetine, an inhibitor of cytosolic protein synthesis. Prior to the addition of labeled methionine, the cells were pretreated with the nucleoside analogs for 6 days. At this time point, methionine was added, and the cells were incubated for a further 3 h before lysis of the cells in denaturing buffer. After being normalized for protein content, the cell lysates were separated by urea–SDS-polyacrylamide gel electrophoresis and subjected to autoradiography (Fig. 4). In the control cells, 11 of 13 mitochondrially coded polypeptides were identified by their apparent molecular weights, and synthesis of these proteins was totally inhibited by chloramphenicol, confirming their mitochondrial origin. In the cells treated with nucleosides, no quantitative differences in polypeptide levels between the untreated control and AZT-, d4T-, or 3TC-treated cells were

found (Fig. 4). In contrast, ddC greatly reduced the total mtDNA-encoded-subunit content, while ddI at this concentration (50 μM) inhibited it to a lesser extent. Quantification of the intensity of the autoradiograms indicated evidence for some selectivity in the extent of inhibition of individual polypeptides, most striking with ATP6, ATP8, and ND3 (Table 1). These data further indicate that where inhibition of mtDNA replication occurs, it has a profound effect on the rate of synthesis of mitochondrially synthesized proteins.

Effects of nucleoside analogs on the respiratory-chain complexes and citrate synthase activities. To determine whether inhibition of mitochondrial protein synthesis resulted in modification of enzyme activity, the effects of these antiretroviral drugs on the functions of isolated enzymatic components of the mitochondria were determined. Mitochondria were isolated from HepG2 cells after a 6-day exposure to 10 and 50 μM AZT, 50 μM AMT and d4T, and 10 μM ddC. The data are summarized in Table 2 and indicate that only in the case of AZT was there a significant effect on enzyme-containing subunits encoded by mtDNA. It is interesting to note that even though ddC, and to some extent ddI, inhibited mitochondrial protein synthesis, this was not reflected in the activities of critical enzymes containing these polypeptide subunits. AZT at 50 μM significantly decreased (by 30%) COX activity while having no significant effect on other mitochondrial complexes. COX activity was completely inhibited by potassium cyanide, excluding the possibility of nonspecific oxidation of cytochrome c. Therefore, the possibility of an interaction between AZT and COX was raised. However, with isolated human liver mitochondria, AZT did not inhibit COX activity at concentrations up to 1 mM. Comparable results were obtained with AMT, AZT monophosphate, and ddC (data not shown). Therefore, AZT-reduced COX activity is not the result of a direct binding of AZT to COX.
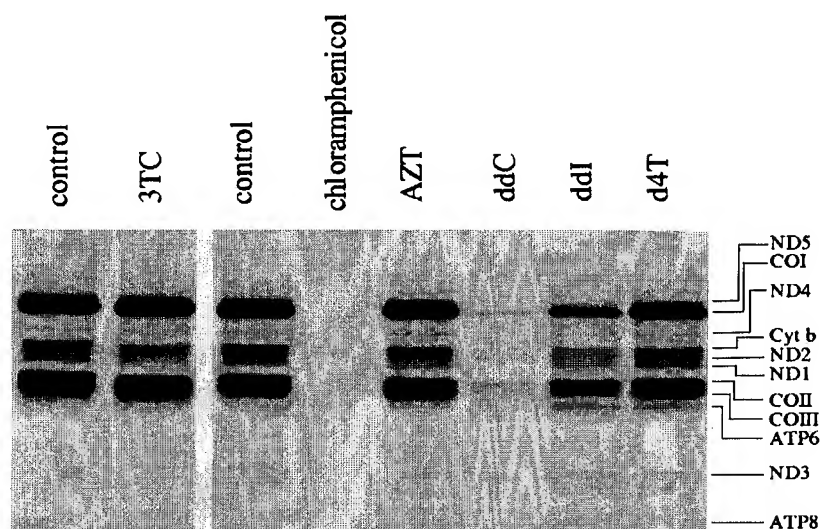
FIG. 4. Autoradiography of radiolabeled mitochondrially coded proteins. After 6 days of treatment with AZT, ddC, ddI, d4T, or 3TC, the medium of HepG2 cells (2.5 × 10⁶/ml) was replaced by the same medium deficient in methionine. Cytoplasmic protein synthesis was inhibited by emetine (200 μg/ml), and mitochondrially coded proteins were labeled by adding 60 μCi of [³⁵S]methionine/ml. To one set of the control cells was added chloramphenicol (200 μg/ml) to inhibit mitochondrial protein synthesis. After 3 h, the cells were washed with PBS and dissolved in 5% SDS–Tris buffer prior to electrophoresis. Electrophoresis and autoradiography were then performed as described previously (17).

Numerous studies have shown that COX is not limiting for oxidative phosphorylation, making it unlikely that inhibition of COX by 30% would significantly affect ATP synthesis (19). However, the data showing increased lactate in the medium suggest a combination of mitochondrial defects or that inhibition of metabolism is occurring at some other point prior to electron transport. To examine the effect of AZT on a critical enzyme in the tricarboxylic acid cycle, the activity of citrate synthase was measured and found to be inhibited by 30% (Table 2).

TABLE 1. mtDNA-encoded polypeptide synthesis after exposure of HepG2 cells to 50 μM AZT, d4T, and 3TC and 10 μM ddC

Mitochondrial polypeptide[a]	%[b]				
	AZT	ddC	ddI	d4T	3TC
ND5 + COI	96	14	52	91	103
ND4	115	45	87	106	100
Cyt b	94	27	66	89	101
ND2	95	27	77	96	97
ND1	82	26	76	99	97
COII	88	22	75	95	99
COIII	99	18	61	90	96
ATP6	92	42	116	117	112
ND3	88	67	139	112	113
ATP8	107	80	166	156	116
8 subunits	96	24	68	93	101
Total subunits	99	33	88	100	104

[a] ND, components of NADH dehydrogenase; CO, components of COX; cyt b, subunit of ubiquinol-COX; ATP, components of ATP synthase.

[b] The relative amount of mtDNA-encoded subunit was determined by scanning the autoradiograph using Bio-Rad imaging densitometer model GS-670. The data are normalized based on the percentage of band density in each sample compared with the respective control and represent the means of two independent experiments. In all cases, the variability was less than 5%.

DISCUSSION

Massive macrovesicular steatosis accompanied by severe lactic acidosis and hepatic failure has been reported following chronic treatment with AZT, ddC, ddI, and d4T (40). Ultrastructural examination of liver autopsy specimens from a patient revealed only enlarged mitochondria after AZT treatment (32). In HIV-infected patients who have developed myopathy after long-term AZT treatment, severe metabolic defects were observed, including accumulation of lipid droplets in myotubes (16). In addition, changes in skeletal muscle mitochondria with swelling of the organelle and disruption of cristae were observed (8, 9, 15, 37).

Since mitochondria exhibit substantial heterogeneity in differentiated cells, different organs and target cells may display different sensitivities. The mitochondrial-morphological evaluation reported here with AZT in HepG2 cells is consistent with the in vivo findings. Similarly, in vitro studies indicated that FIAU has a profound effect on lipid accumulations in both hepatocytes and myocytes (14, 36). In our study, AZT and other nucleoside analogs (ddC, ddI, d4T, and 3TC) did not lead to accumulation of lipid droplets in HepG2 cells even with extended exposure of up to 14 days, which is consistent with the reports of exposure of this duration. This may reflect the possibility that AZT and FIAU act on different target(s) in mitochondria, particularly those related to lipid metabolism.

The concentration of nucleosides that the mitochondria would be exposed to remains an area of some uncertainty; however, some realistic estimates can be made based on in vitro experiments. Interestingly, AZT was shown to be able to accumulate in the mitochondrial matrix, reaching a fourfold-higher concentration than in the surrounding medium (1). Extrapolating these data to the studies shown here would result in a concentration range of 30 to 50 μM in the mitochondria. Since accumulation of AZT in the mitochondria is also time dependent, this could represent a low estimate and is consistent with the dosing regimens used here to model acute

TABLE 2. Effects of antiviral nucleoside analogs on the activities of respiratory-chain enzyme complexes[a]

Drug (concn [μM])	Activity[b]				
	Complex I	Complex II	Complex III	Complex IV	Citrate synthase
AZT (10)	101.2 ± 14.8	109.8 ± 6.4	107.9 ± 19.6	85.1 ± 7.8	ND
AZT (50)	103.3 ± 14.9	87.5 ± 9.2	94.2 ± 11.2	71.7 ± 9.1[c]	70.8 ± 10.8[d]
AMT (50)	103.0 ± 8.5	96.5 ± 4.5	95.6 ± 24.4	93.7 ± 8.2	ND
ddC (1)	110.6 ± 6.6	112.5 ± 1.0	92.9 ± 20.1	100.9 ± 2.2	ND
ddC (10)	107.9 ± 7.6	104.4 ± 17.1	97.7 ± 9.5	88.2 ± 12.0	98.4 ± 8.9
d4T (50)	108.2 ± 15.0	112.3 ± 5.6	98.2 ± 0.6	113.7 ± 8.8	ND

[a] HepG2 cells were exposed to various concentrations of nucleoside analogs for 6 days. Mitochondria were then extracted as described in Materials and Methods. The mean value of complex I enzyme activity was 125.3 ± 32.4 nmol/min/mg of mitochondrial protein. The mean value of complex II enzyme activity was 157.5 ± 28.9 nmol/min/mg of mitochondrial protein. Complex III activity was 0.513 ± 0.11 first-order rate constant/mg of mitochondrial protein, and complex IV activity was 0.435 ± 0.122 first-order rate constant/mg of mitochondrial protein. Citrate synthase activity was 0.378 ± 0.05 nmol/min/mg of protein.

[b] Data are presented as percentages ± standard deviations ($n \geq 3$) of the activity of the control in the same experiment. ND, not determined.

[c] $P < 0.005$.

[d] $P < 0.05$.

and chronic exposure to the drug. Recently, similar results were obtained with cultured human muscle cells, indicating that AZT, ddC, and ddI at 1 mM significantly decreased complex II and IV activity (2). Concentrations of AZT above 5 mM were required for 50% inhibition of COX activity. These results are difficult to interpret, since the concentrations used were 100- to 500-fold higher than pharmacologically relevant AZT levels, even accounting for mitochondrial uptake.

In this study, the nucleoside found to induce lactate production to the greatest extent in HepG2 cells was AZT (Table 3). While accepting the limitations inherent in using cell cultures to investigate the cytotoxic effects of these compounds, we hypothesize that this acute response is best explained by the inhibition of both citrate synthase and COX. Citrate synthase is a key enzyme of the citric acid cycle and is an excellent marker for mitochondrial functionality. The citric acid cycle is the final common pathway for the oxidation of amino acids, fatty acids, and carbohydrates. Interference by AZT with citrate synthase would result in a low ATP/ADP ratio and favor the alternative anaerobic metabolic pathway of pyruvate, leading to an enhanced formation of lactic acid. A deficiency of COX has been associated with a number of human diseases, including Leigh syndrome, chronic progressive ophthalmoplegia, and fatal and benign infantile mitochondrial myopathy (42). COX deficiency can be caused by mutations in nuclear DNA and/or mtDNA (42). AZT has been shown to inhibit COX, while no mtDNA depletion could be detected. Incorporation of AZT into host DNA and inhibition of cellular DNA polymerases and exonucleases, as well as inhibition of hemoglobin synthesis, have been suggested as possible mechanisms for AZT-induced hematological toxicity (12, 39, 44). Therefore, the decreased COX activity may be a result of mutation of nuclear DNA induced by AZT. Previously, it has also been reported that AZT inhibited the mitochondrial ATP synthase

after the exposure of cells to AZT (26). Multiple sites of inhibition by AZT appear to result in mitochondrial dysfunction, causing hepatic failure.

Compared to AZT, FIAU at 25 μM had no inhibitory effect on respiratory-chain enzyme activities and mtDNA-encoded-subunit synthesis (data not shown). The FIAU-related mitochondrial dysfunction appears to have a different mechanism than that of AZT-related dysfunction.

Three currently used anti-HIV drugs, ddC, ddI, and d4T, have been reported to induce pancreatitis, neuropathy, and liver toxicity in patients with AIDS through mitochondrial dysfunction (6, 20, 45). However, each drug displays a different potency in its effect on mitochondria. ddC and ddI increased lactic acid to a lesser extent than AZT and AMT, while no changes were observed with d4T and 3TC treatment. ddC and ddI were shown to profoundly affect mitochondrial structure, probably as a consequence of their inhibition of mtDNA replication and mtDNA-encoded polypeptide synthesis. The mitochondrial effects of d4T appeared to be cell type specific. No mtDNA depletion was observed with d4T in PC-12 and HepG2 cells, while mtDNA synthesis of CEM cells was inhibited by d4T at concentrations less than 10 μM (11). In the present study, d4T did not increase the lactic acid level, had no effect on mtDNA-encoded-polypeptide synthesis, and did not reduce respiratory-chain enzyme activities, suggesting that other, unidentified cellular target(s) may be involved in the d4T-induced mitochondrial toxicity. Although complexes I, III, and IV contain subunits encoded by both the mitochondrial and nuclear DNAs, most of the subunits are encoded in the nucleus, synthesized in the cytosol, and imported into mitochondria. The effects of nucleoside analogs on the synthesis of nuclear-DNA-encoded polypeptides remain unknown. Nevertheless, deficiencies in mtDNA-encoded subunits caused by ddC and ddI may provide a molecular basis for the occurrence of the drug-

TABLE 3. Summary of effects of anti-HIV nucleoside analogs on treated HepG2 cells

Effect	Response[a]				
	AZT	ddC	d4T	ddI	3TC
Increased lactic acid levels	++	+	−	+	−
Mitochondrial morphological changes	Enlarged mitochondria	Loss of crisitae	−	Loss of cristae	−
Inhibition of mtDNA	−	+++	−	++	−
Inhibition of mitochondrial polypeptides	−	+++	−	++	−
Inhibition of COX and citrate synthase	+	−	ND	ND	ND

[a] ND, not determined.

induced hepatotoxicity. During chronic exposure, it is postulated that a slow but progressive depletion of mitochondrially coded proteins will occur, resulting in emerging defects in electron transport as the period of treatment increases. This would imply that a synergistic effect on respiratory function caused by combination therapy with nucleoside analogs could occur, although no direct clinical evidence indicates that this is the case.

In summary, the results from the present study indicated that AZT selectively inhibited COX and citrate synthase activities of HepG2 cells, while ddC, d4T, and AMT had no such effects. This AZT-related inhibition may reduce the content of intracellular ATP, resulting in the compensating enhancement of the glycolytic pathway, and finally enhancing the production of lactate. ddC and ddI significantly inhibited mtDNA content and mitochondrial protein synthesis, while none of the complex (I to IV) activities were inhibited by ddC (Table 3). This has been noted before in other models related to exposure to mitochondrial toxins (31, 38). This can be reconciled if the slow turnover of mitochondrial proteins is a critical factor leading to maintenance of enzymatic activity during inhibition of new protein synthesis. It is not clear why the myocyte is more sensitive to nucleoside-dependent mitochondrial dysfunction than hepatocytes, given the expectation that the reserve capacity of the muscle cell should be higher than that of the liver. However, little is known of the relative turnover of mitochondrial proteins in these two tissues, and in fact, inherited mitochondrial defects are more often manifested in muscle tissue than in the liver (27). This is likely to be related to differential reliance on oxidative phosphorylation of the two tissues. The clinical relevance of these findings has yet to be established, but one may conclude that the concepts of thresholds and reserve capacity critical to our understanding of mitochondrial function should be extended to the replication and synthesis of proteins coded for by the mitochondrial genome.

REFERENCES

1. Barile, M., D. Valenti, S. Passarella, and E. Quagliariello. 1997. 3'-Azido-3'-deoxythmidine uptake into isolated rat liver mitochondria and impairment of ADP/ATP translocator. Biochem. Pharmacol. 53:913–920.
2. Benbrik, E., P. Chariot, S. Bonavaud, M. Ammi-Said, E. Frisdal, C. Rey, R. Gherardi, and G. Barlovatz-Meimon. 1997. Cellular and mitochondrial toxicity of zidovudine (AZT), didanosine (ddI) and zalcitabine (ddC) on cultured human muscle cells. J. Neurol. Sci. 149:19–25.
3. Birch-Machin, M., S. Jackson, R. Singh Kler, and D. M. Turnbull. 1993. Study of skeletal muscle mitochondrial dysfunction, p. 51–69. In L. H. Lash and D. P. Jones (ed.), Methods in toxicology: mitochondrial dysfunction. Academic Press, San Diego, Calif.
4. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248–254.
5. Bridges, E. G., A. Faraj, and J.-P. Sommadossi. 1993. Inhibition of mammalian DNA polymerase-associated 3' to 5' exonuclease activity by 5'-monophosphates of 3'-azido-3'-deoxythymidine and 3'-amino-3'-deoxythymidine. Biochem. Pharmacol. 45:1571–1576.
6. Browne, M. J., K. H. Mayer, S. B. Chafee, M. N. Dudley, M. R. Posner, S. M. Steinberg, K. K. Graham, S. M. Geletko, S. H. Zinner, S. L. Denman, L. M. Dunkle, S. Kaul, C. Mclaren, G. Skowron, N. M. Kouttab, T. A. Kennedy, A. B. Weitberg, and G. A. Curt. 1993. 2',3'-Didehydro-3'-deoxythymidine (d4T) in patients with AIDS or AIDS-related complex: a phase I trial. J. Infect. Dis. 167:21–29.
7. Burchell, A., R. Hume, and B. Burchell. 1988. A new microtechnique for the analysis of the human hepatic microsomal glucose-6-phosphatase system. Clin. Chim. Acta 173:183–191.
8. Casademont, J., A. Barrientos, J. M. Grau, E. Pedrol, X. Estivill, A. Urbano-Marquez, and V. Nunes. 1996. The effect of zidovudine on skeletal muscle mtDNA in HIV-1 infected patients with mild or no muscle dysfunction. Brain 119:1357–1364.
9. Chariot, P., F. Le Maguet, F. J. Authier, D. Labes, F. Poron, and R. Gherardi. 1995. Cytochrome c oxidase deficiency in zidovudine myopathy affects perifascicular muscle fibres and arterial smooth muscle cells. Neuropathol. Appl. Neurobiol. 21:540–547.
10. Chariot, P., I. Monnet, and R. Gherardi. 1993. Cytochrome c oxidase reac-
11. Chen, C. H., M. Vazquez-Padua, and Y. C. Cheng. 1991. Effect of anti-human immunodeficiency virus nucleoside analogs on mitochondrial DNA and its implication for delayed toxicity. Mol. Pharmacol. 39:625–628.
12. Cheng, Y. C., C. H. Chen, M. A. Vazquez-Padua, and M. C. Starnes. 1990. DNA polymerases versus HIV reverse transcriptase in AIDS therapy. Ann. N. Y. Acad. Sci. 616:217–223.
13. Cui, L., L. Locatelli, M. Y. Xie, and J.-P. Sommadossi. 1997. Effect of nucleoside analogs on neurite regeneration and mitochondrial DNA synthesis in PC-12 cells. J. Pharmacol. Exp. Ther. 280:1228–1234.
14. Cui, L., S. Yoon, R. S. Schinazi, and J.-P. Sommadossi. 1995. Cellular and molecular events leading to mitochondrial toxicity of 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil in human liver cells. J. Clin. Investig. 95:555–563.
15. Dalakas, M. C., M. E. Leon-Monzon, I. Bernardini, W. A. Gahl, and C. A. Jay. 1994. Zidovudine-induced mitochondrial myopathy is associated with muscle carnitine deficiency and lipid storage. Ann. Neurol. 35:482–487.
16. Dalakas, M. C., I. Illa, G. H. Pezeshkpour, J. P. Laukaita, B. Cohen, and J. L. Griffin. 1990. Mitochondrial myopathy caused by long-term zidovudine therapy. N. Engl. J. Med. 322:1098–1105.
17. Darley-Usmar, V. M., R. A. Capaldi, S. Takamiya, F. Millett, M. T. Wilson, F. Malatesta, and P. Sarti. 1987. Reconstitution and molecular analysis of the respiratory chain, p. 113–126. In V. M. Darley-Usmar, D. Rickwood, and M. T. Wilson (ed.), Mitochondria: a practical approach. IRL press, Oxford, England.
18. Davey, G. P., L. Canevari, and J. B. Clark. 1997. Threshold effects in synaptosomal and nonsynaptic mitochondria from hippocampal CA1 and paramedian neocortex brain regions. J. Neurochem. 69:2564–2570.
19. Davey, G. P., S. Peuchen, and J. B. Clark. 1998. Energy thresholds in brain mitochondria. Potential involvement in neurodegeneration. J. Biol. Chem. 273:12753–12757.
20. Dubinsky, R. M., R. Yarchoan, M. Dalakas, and S. Broder. 1989. Reversible axonal neuropathy from the treatment of AIDS and related disorders with 2',3'-dideoxycytidine (ddC). Muscle Nerve 12:856–860.
21. Dusheiko, G. M. 1994. Fialuridine toxicity: new hopes and false downs. Int. Antiviral News 2:22–23.
22. Fortgang, I. S., P. C. Belitsos, R. E. Chaisson, and R. D. Moore. 1995. Hepatomegaly and steatosis in HIV-infected patients receiving nucleoside analog antiretroviral therapy. Am. J. Gastroenterol. 90:1433–1436.
23. Freiman, J. P., K. E. Helfert, M. R. Hamrell, and D. S. Stein. 1993. Hepatomegaly with severe steatosis in HIV-seropositive patients. AIDS 7:379–385.
24. Furman, P. A., J. A. Fyfe, M. H. St. Clair, K. Weinhold, J. L. Rideout, G. A. Freeman, S. N. Lehrman, D. P. Bolognesi, S. Broder, H. Mitsuya, and D. W. Barry. 1986. Phosphorylation of 3'-azido-3'-deoxythymidine and selective interaction of the 5'-triphosphate with human immunodeficiency virus reverse transcriptase. Proc. Natl. Acad. Sci. USA 83:8333–8337.
25. Gray, N. M., C. L. Marr, C. R. Penn, J. Cameron, and R. C. Bethell. 1995. The intracellular phosphorylation of (−)-2'-deoxy-3'-thiacytidine (3TC) and the incorporation of 3TC 5'-monophosphate into DNA by HIV-1 reverse transcriptase and human DNA polymerase gamma. Biochem. Pharmacol. 50:1043–1051.
26. Hobbs, G. A., S. A. Keilbaugh, P. M. Rief, and M. V. Simpson. 1995. Cellular targets of 3'-azido-3'-deoxythymidine: an early (non-delayed) effect on oxidative phosphorylation. Biochem. Pharmacol. 50:381–390.
27. Kovalenko, S. A., G. Kopsidas, J. Kelso, F. Rosenfeldt, and A. W. Linnane. 1998. Tissue-specific distribution of multiple mitochondrial DNA rearrangements during human aging. Ann. N. Y. Acad. Sci. 854:171–181.
28. Martin, J. L., C. E. Brown, N. Matthews-Davis, and J. E. Reardon. 1994. Effects of antiviral nucleoside analogs on human DNA polymerases and mitochondrial DNA synthesis. Antimicrob. Agents Chemother. 38:2743–2749.
29. Mhiri, C., M. Baudrimont, G. Bonne, C. Geny, F. Degoul, C. Marsac, E. Roullet, and R. Gherardi. 1991. Zidovudine myopathy: a distinctive disorder associated with mitochondrial dysfunction. Ann. Neurol. 29:606–614.
30. Mitsuya, H., and S. Broder. 1986. Inhibition of the in vitro infectivity and cytopathic effect of human T-lymphotrophic virus type III/lymphadenopathy-associated virus (HTLV-III/LAV) by 2',3'-dideoxynucleosides. Proc. Natl. Acad. Sci. USA 83:1911–1915.
31. Miyako, K., Y. Kai, T. Irie, K. Takeshige, and D. Kang. 1997. The content of intracellular mitochondrial DNA is decreased by 1-methyl-4-phenylpyridinium ion (MPP+). J. Biol. Chem. 272:9605–9608.
32. Olano, J. P., M. J. Borucki, J. W. Wen, and A. K. Haque. 1995. Massive hepatic steatosis and lactic acidosis in a patient with AIDS who was receiving zidovudine. Clin. Infect. Dis. 21:973–976.
33. Peters, B. S., J. Winer, D. N. Landon, A. Stotter, and A. J. Pinching. 1993. Mitochondrial myopathy associated with chronic zidovudine therapy in AIDS. Q. J. Med. 86:5–15.
34. Rickwood, D., M. T. Wilson, and V. M. Darley-Usmar. 1987. Isolation and characteristics of intact mitochondria, p. 6. In V. M. Darley-Usmar, D.

tion improves histopathological assessment of zidovudine myopathy. Ann. Neurol. 34:561–565.

Rickwood, and M. T. Wilson (ed.), Mitochondria: a practical approach. IRL Press, Oxford, England.

35. **Robinson, J. B., L. G. Brent, B. Sumegi, and P. A. Srere.** 1987. An enzymatic approach to the study of the Krebs tricarboxylic acid cycle, p. 160–161. *In* V. M. Darley-Usmar, D. Rickwood, and M. T. Wilson (ed.), Mitochondria: a practical approach. IRL Press, Oxford, England.

36. **Semino-Mora, C. M., M. Leon-Monzon, and M. C. Dalakas.** 1997. Mitochondrial and cellular toxicity induced by fialuridine in human muscle in vitro. Lab. Investig. **76:**487–495.

37. **Sinnwell, T. M., K. Sivakumar, S. Soueidan, C. Jay, J. A. Frank, A. C. McLaughlin, and M. C. Dalakas.** 1995. Metabolic abnormalities in skeletal muscle of patients receiving zidovudine therapy observed by 31P in vivo magnetic resonance spectroscopy. J. Clin. Investig. **96:**126–131.

38. **Slipetz, D. M., J. R. Aprille, P. R. Goodyer, and R. Rozen.** 1991. Deficiency of complex III of the mitochondrial respiratory chain in a patient with facioscapulohumeral disease. Am. J. Hum. Genet. **48:**502–510.

39. **Sommadossi, J.-P., R. Carlisle, and Z. Zhou.** 1989. Cellular pharmacology of 3′-azido-3′-deoxythymidine with evidence of incorporation into DNA of human bone marrow cells. Mol. Pharmacol. **36:**9–14.

40. **Styrt, B., and J. P. Freiman.** 1995. Hepatotoxicity of antiviral agents. Gastroenterol. Clin. N. Am. **24:**839–852.

41. **Sundar, K., M. Suarez, P. E. Banogon, and J. M. Shapiro.** 1997. Zidovudine-induced fatal lactic acidosis and hepatic failure in patients with acquired immunodeficiency syndrome: report of two patients and review of the literature. Crit. Care Med. **25:**1425–1430.

42. **Taanman, J. W.** 1997. Human cytochrome c oxidase: structure, function, and deficiency. J. Bioenerg. Biomembr. **29:**151–163.

43. **Touchette, N.** 1993. HBV-drug deaths prompt restudy of similar antivirals. J. NIH Res. **5:**33–35.

44. **Weidner, D. A., and J.-P. Sommadossi.** 1990. 3′-Azido-3′-deoxythymidine inhibits globin gene transcription in butyric acid-induced K562 human leukemia cells. Mol. Pharmacol. **38:**797–804.

45. **Yarchoan, R., J. M. Pluda, R. V. Thomas, H. Mitsuya, P. Brouwers, K. M. Wyvill, N. Hartman, D. G. Johns, and S. Broder.** 1990. Long-term toxicity/activity profile of 2′,3′-dideoxyinosine in AIDS or AIDS-related complex. Lancet **336:**526–529.